

## Analytical Artifacts in Radioimmunoassay of L-Thyroxine in Human Milk

**J. Mallol, M. J. Obregón, and G. Morreale de Escobar<sup>1</sup>**

Previous results are contradictory regarding the concentration of thyroxine in human milk. Using a sensitive radioimmunoassay, we have found a lack of parallelism between the standard curve for thyroxine and the curve for serial dilutions of whole human milk, skimmed milk, or ethanol extracts of milk. Nonspecific binding also indicated the presence of analytical artifacts. Thus we have separated thyroxine from other milk components by means of a strongly basic Bio-Rad anion-exchange resin with quaternary ammonium exchange groups attached to a styrene divinyl benzene copolymer lattice, radioimmunoassaying the fractions eluted with an equivolume mixture of acetic acid and water. Parallelism with the standard curve was good, and results were the same whether or not the resin eluate was further purified by paper chromatography. The range of thyroxine concentration in 21 samples of human milk was 0.29–2.00  $\mu\text{g/L}$  (mean 0.71, SD 0.40,  $\mu\text{g/L}$ ). Such concentrations are unlikely to afford protection to the developing brain of a breast-fed athyreotic baby, as previously claimed.

**Additional Keyphrases:** *reference interval · disorders of thyroxine production · hypothyroidism · newborns · chromatography, paper · chromatography, anion-exchange*

Montalvo et al. (1) and Štrbák et al. (2) reported, respectively, 7–96 and 15–130  $\mu\text{g}$  of thyroxine ( $\text{T}_4$ ) per liter of human milk, as determined with competitive binding assays of whole milk, skimmed milk, or ethanol extracts of milk. Sack et al. (3) and Tenore et al. (4) reported values of 4–70 and 13–80  $\mu\text{g}$  of  $\text{T}_4$  per liter of human milk, respectively, as measured by radioimmunoassay (RIA).<sup>2</sup> Such concentrations of  $\text{T}_4$  might afford a considerable degree of protection to the brain of hypothyroid babies. Indeed, Tenore et al. (4) reported improved mental development in breast-fed, as compared with formula-fed, athyreotic babies and attributed this finding to the higher  $\text{T}_4$  content of human milk.

These findings contrast with those of Varma et al. (5), who reported the concentration of  $T_4$  as determined by RIA to be  $<5 \mu\text{g/L}$  in 83 out of 88 samples of human milk. Sato and Suzuki (6) also found  $T_4$  concentrations of  $<5 \mu\text{g/L}$  in 100 samples of human milk, and in trypsin digests of 20 samples. These results are consistent with the report of Tsuyusaki et al. (7), who found that ethanol extracts of human milk had no biological activity as measured by a goiter prevention bioassay.

The aim of the present work was to ascertain which of these contradictory reports is correct. We used a highly sensitive RIA, developed for serum T<sub>4</sub> by Weeke and Ørskov (8). None of the 21 samples we have studied so far contains >2 µg of T<sub>4</sub>.

per liter; thus we conclude that the lower values reported are more nearly correct, but still high.

## Materials and Methods

***T<sub>4</sub> RIA.*** We used the method of Weeke and Ørskov (8) described for assay of T<sub>4</sub> in human serum, as modified by Obregón et al. (9), in which RIA buffer is used as the solvent for standards used in preparing the T<sub>4</sub> standard curve and polyethylene glycol 6000 (PEG) is used to separate free from antibody-bound labeled T<sub>4</sub>. <sup>125</sup>I-labeled T<sub>4</sub> (T<sub>4</sub><sup>\*</sup>) of high specific activity (2000 Ci/g, or higher) was obtained by the method of Weeke and Ørskov (10) as modified by Kjeld et al. (11). The sensitivity is 5 pg of T<sub>4</sub> per tube, 0.5 μL of human serum being sufficient.

In brief, the  $T_4$  standards, prepared in 50  $\mu$ L of RIA phosphate buffer (40 mmol/L, pH 8.0, and containing 0.6 mmol of thimerosal and 0.5 g of bovine serum albumin per liter), are pipetted into test tubes. Milk samples are pipetted in quadruplicate at each dilution and diluted to 50  $\mu$ L with the RIA buffer. Three of these quadruplicate tubes receive 100  $\mu$ L of  $T_4$  antiserum at a 20 000-fold dilution, and the fourth receives 100  $\mu$ L of buffer. Thus there is an individual "blank" for each triplicate of each dilution of a milk sample. Approximately 7000 cpm of  $T_4^*$  is added, in 100  $\mu$ L of RIA buffer. After 16- to 20-h incubation at room temperature, 1.5 mL of RIA buffer containing 200 g of PEG and 50 mL of normal calf serum per liter is added and the tubes and contents are vigorously vortex-mixed for 20 s. After centrifugation at 2000 rpm for 45 min, the supernatant fluid is aspirated and the radioactivity in the precipitate is counted. RIA buffer "blanks" are used to determine the nonspecifically bound  $T_4^*$  for tubes containing  $T_4$  standards, whereas individual "blanks" are used for milk samples.

**Sample-preparation procedure A.** Whole milk was obtained from volunteers at different times after delivery and stored at  $-20^{\circ}$  until used.

Unless whole milk is used directly for the RIA, add about 5000 cpm of  $T_4^*$  to 1.0 mL of whole milk. After 2 h at room temperature, centrifuge the radiolabeled milk for 45 min at 2000 rpm, or extract it with absolute ethanol. In the first case, separate the skimmed milk from the fat layer at 4 °C. In the second case, add either the same volume of ethanol (1:2 EtOH extract) or nine volumes of ethanol (1:10 EtOH extracts) to whole or skimmed milk, centrifuge, and use the supernate for analysis. Count the radioactivity in aliquots of whole and skimmed milk and of EtOH extracts to determine the  $T_4^*$  partition and extraction coefficients after the usual corrections for counting geometry, efficiency, etc. After centrifugation of whole milk, dissolve the fat recovered in a volume of EtOH corresponding to the initial volume of whole milk.

Assay whole or skimmed milk, or EtOH extracts, at three dilutions. Evaporate EtOH extracts to dryness at room temperature under a stream of air. The radioactivity of these samples was <200 cpm per tube. Follow the above T<sub>4</sub> RIA procedure.

Aliquot volumes indicated in Tables and Figures and in the text are given in "equivalents" of whole or skimmed milk. For example, a "50- $\mu$ L aliquot" of a 1:2 EtOH extract means that 100  $\mu$ L of EtOH was actually evaporated for the RIA; in the

Departamento de Endocrinología Experimental, Instituto G. Martínez, C.S.I.C y Facultad Autónoma de Medicina, Arzobispo Morcillo 4. Madrid-34. Spain.

<sup>1</sup> Address correspondence to this author.

<sup>2</sup> Nonstandard abbreviations: T<sub>4</sub>, L-thyroxine; T<sub>4</sub><sup>\*</sup>, radiolabeled T<sub>4</sub>; PEG, polyethylene glycol.

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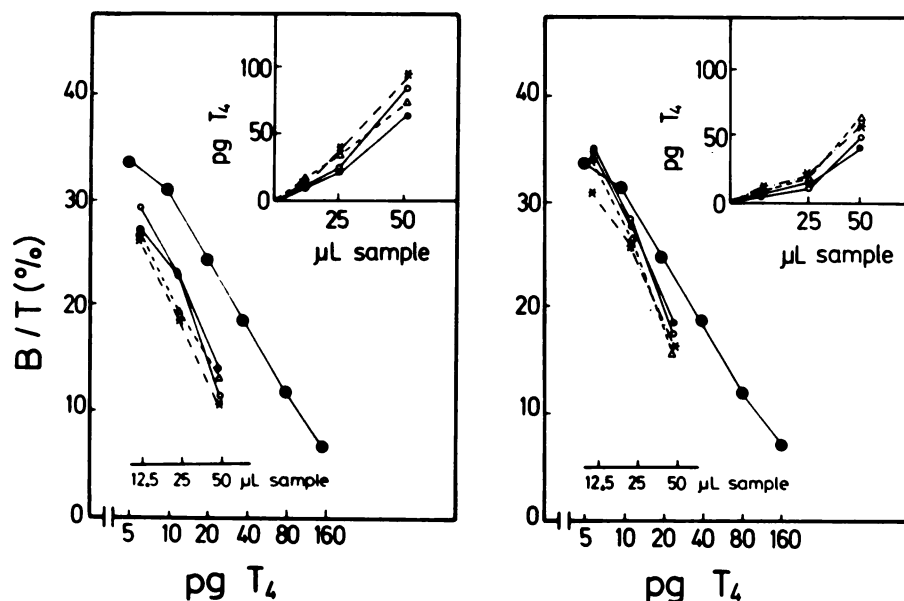


Fig. 1. Serial dilution curves obtained with (left) whole milk and (right) skimmed milk, as compared with the standard curve prepared with use of RIA buffer

*Insets:* the apparent  $T_4$  contents as a function of the volume of sample used for the RIA. As may be seen, the data usually do not fall on a straight line passing through the origin. Different symbols are used to identify samples from different milk donors. Results are only shown for four donors, but samples for 15 were tested. Most of these 15 samples did not give dilution curves that paralleled the standard curve

case of milk fat, a "50- $\mu$ L aliquot" would indicate the fat contained in 50  $\mu$ L of the original whole milk.

**Sample-preparation procedure B: isolation of  $T_4$  by chromatography on Bio-Rad columns.** Add  $T_4^*$  (7000 cpm) to 1.5 mL of whole milk (or RIA buffer for the "blank" column). After 1 h at room temperature, pass the mixture through a 1.5-mL column of Bio-Rad 1  $\times$  2 (200–400 mesh, in chloride form) (12) equilibrated with acetate buffer (0.2 mol/L, pH 7.0). Wash the column with 2 mL of the same buffer and then with 4 mL of EtOH; this removes most of the milk proteins and lipids. Then wash with 2 mL of acetate buffer (0.2 mol/L) at pH 4.0 and 2 mL at pH 3.0, followed by 2 mL of 18 mmol/L (10 mL/L) and 2 mL of 610 mmol/L (350 mL/L) acetic acid. Discard all these eluates. Then pass seven or eight 0.5-mL fractions of 880 mmol/L (500 mL/L) acetic acid through the column, collect the eluates and count the radioactivity of each. Pool the two or three fractions containing the highest radioactivity to obtain "eluate 1." This contains more than half of the  $T_4^*$  that was added to the milk sample and is clear and colorless, even when the milk sample has a high lipid content. Count the radioactivity in an aliquot to determine the analytical recovery of  $T_4^*$ . Pipet other aliquots (usually 40 and 80  $\mu$ L) in quadruplicate into RIA tubes and evaporate them. Add 50  $\mu$ L of RIA buffer and follow the  $T_4$  RIA procedure, except add more  $T_4^*$  than usual (8000–9000 cpm per tube) to minimize the influence of the 200–300 cpm of  $T_4^*$  in the eluate-1 aliquots. Calculate the concentration of  $T_4$  in the initial milk sample from the amount of  $T_4$  found in the aliquot and the recovery of  $T_4^*$  in a known volume of eluate 1.

**Sample-preparation procedure C: isolation of  $T_4$  by procedure B and paper chromatography.** To validate procedure B, evaporate another aliquot of eluate 1, dissolve the residue in small volumes of methanol/ammonia (99/1 by vol), and transfer the solution to Whatman 3MM paper strips, 2.5-cm wide, previously spotted with 50  $\mu$ L of EtOH containing propylthiouracil, 10 mmol/L, to protect  $T_4$  from artifactual de-iodination. Submit the strips to descending chromatography in 2-methylbutanol/hexane/ammonia, 2 mol/L (5/1/6 by vol), as described by Bellabarba et al. (13). Dry the chromatograms, cut them into strips 1-cm wide, and count the radioactivity. Pool the two or three strips containing the

highest  $T_4^*$  content and elute them with 2.0 mL of methanol/ammonia ("eluate 2"). Count an aliquot for  $T_4^*$  recovery. Pipet other aliquots (usually 50, 100, and 200  $\mu$ L) in quadruplicate into RIA tubes and evaporate at room temperature under a stream of air. Perform the RIA and calculate the concentration of  $T_4$  in milk, as indicated for procedure B.

**Miscellanea:** The Bio-Rad resin was washed before the columns were prepared. It was first suspended in 1.2 mol/L acetic acid and then washed several times with distilled water until the pH was 3–4. It then was washed with acetate buffer (0.2 mol/L, pH 7.0). A resin slurry was poured into 2-mL plastic syringes (9 mm i.d.) up to the 1.5-mL mark. The resin was prevented from passing through the syringe by a 6.3-mm dot of Whatman 3 MM paper. The acetate buffer was drained just before the milk sample was added.

In some cases, *Staphylococcus aureus* was used instead of PEG. A 100 g/L suspension of heat- and formaldehyde-killed bacteria, prepared as described by Kessler (14), was kindly obtained for us by Dr. I. Gil from a protein A-rich Cowan strain. We diluted 15  $\mu$ L of the suspension to 500  $\mu$ L in pH 8

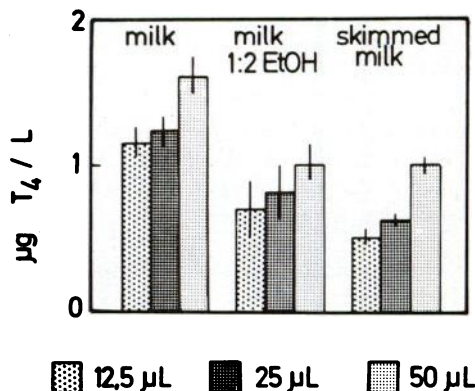


Fig. 2. The apparent  $T_4$  concentrations found on using different aliquots of whole milk, skimmed milk, or 1:2 EtOH extracts of whole milk

The apparent concentration was not independent of the sample volume. The same was true of 1:2 EtOH extracts of skimmed milk or 1:10 EtOH extracts of whole milk. Data are means  $\pm$  SEM of the same samples used for Fig. 1

**Table 1. Assayed Concentrations of  $T_4$  in Whole Milk, Skimmed Milk, and Milk Fat (col. A), and Calculated  $T_4$  Concentrations in Whole Milk (col. B) <sup>a</sup>**

Sample in RIA tube	A: $T_4$ , pg/50 $\mu$ L <sup>b</sup>	B: $T_4$ , $\mu$ g/L <sup>c</sup>
Whole milk	80 (6)	1.60 (0.12)
1:10 EtOH extract	71 (7)	1.57 (0.16)
1:2 EtOH extract	49 (8) <sup>d</sup>	1.07 (0.17) <sup>d</sup>
Skimmed milk	50 (3) <sup>d</sup>	1.42 (0.09)
1:2 EtOH extract	52 (16) <sup>d</sup>	1.48 (0.41)
Fat, EtOH extract	48 (7) <sup>d</sup>	3.22 (0.58) <sup>d</sup>

<sup>a</sup> Data are means (and SEM) for samples from the same donors as for Figures 1 and 2. <sup>b</sup> Amount of  $T_4$  found in the RIA tubes, for 50  $\mu$ L of whole or skimmed milk, the equivalent volumes of EtOH extracts, or the fat corresponding to 50  $\mu$ L of whole milk. <sup>c</sup> Calculated from the  $T_4$  data in column A and the individual  $T_4$  partition and extraction coefficients. <sup>d</sup> Significantly ( $p < 0.05$  or better) different from whole milk, as calculated by the paired  $t$ -test.

phosphate buffer, added this to the tubes, and centrifuged at 2000 rpm for 45 min. The supernate was aspirated and radioactivity in the pellet was counted.

**Statistical analysis.** Mean values and SEM were calculated, and Student's  $t$ -test was performed, as described by Snedecor (15). The paired  $t$ -test was used to evaluate the significance of differences between results obtained by different procedures, to account for variability in milk from different donors.

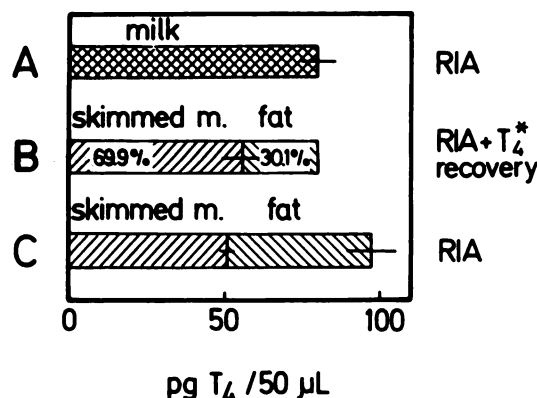
## Results

If the concentrations of  $T_4$  in human milk were about the same as those in serum (1-4), 0.5 to 1.0  $\mu$ L of whole milk should suffice for determination of  $T_4$  with the present RIA (8, 9). But we found that a larger sample volume was necessary before we observed displacement of  $T_4^*$  in the RIA; when we used more sample, however, there was no parallelism between the  $T_4$  standard curve and serial dilutions of most (though not all) of the 15 samples of human milk tested. That is to say, the  $T_4$  concentration was not independent of the volume of sample used in the RIA. Moreover, this problem was not solved by using skimmed milk samples or evaporated EtOH extracts of whole or skimmed milk. This point is illustrated in Figures 1 and 2 for four of the samples tested.

**Table 2. Binding of  $T_4^*$  in the Absence of Anti- $T_4$  Antiserum in RIA Tubes Containing Buffer or Samples of Human Milk <sup>a</sup>**

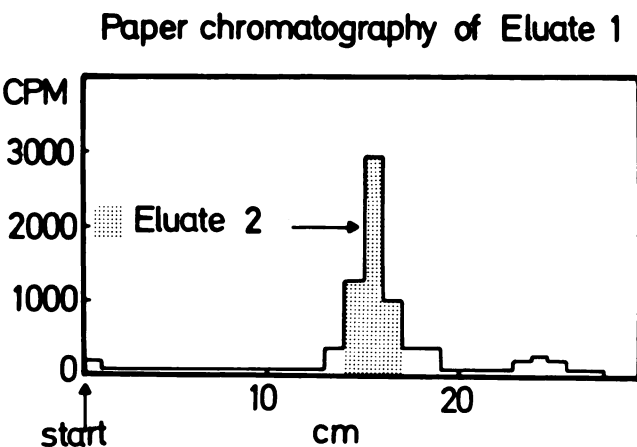
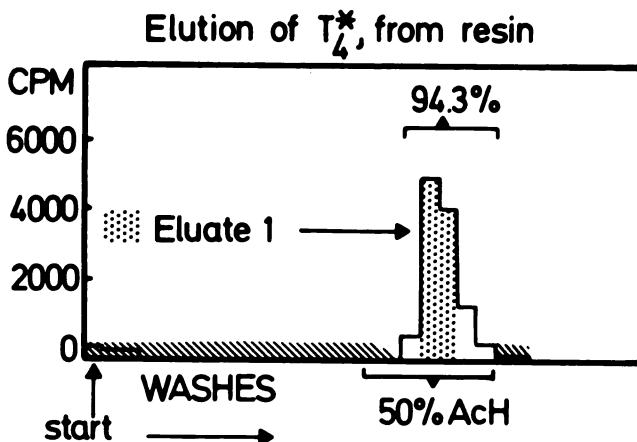
Sample	% of $T_4^*$ in the precipitate	
	A	B
RIA buffer <sup>b</sup>	5.2 (0.3)	10.1 (0.3)
Whole milk	5.9 (0.1)	19.7 (0.8) <sup>c</sup>
1:10 EtOH extract	7.8 (0.6) <sup>c</sup>	19.9 (1.2) <sup>c</sup>
1:2 EtOH extract	8.5 (0.1) <sup>c</sup>	29.1 (5.3) <sup>c</sup>
Skimmed milk	5.1 (1.3)	13.2 (3.1)
1:2 EtOH extract	7.8 (0.5) <sup>c</sup>	16.2 (3.2) <sup>c</sup>
Milk fat, EtOH extract	7.3 (0.6) <sup>c</sup>	46.3 (2.5) <sup>c</sup>

<sup>a</sup> Data are expressed as percentages of the total  $T_4^*$  added, and are means (and SEM) of four samples (same as used for previous Figures and Table). Data in column A were obtained by using PEG, those in column B by using *S. aureus* to separate free from bound  $T_4^*$ . <sup>b</sup> The total binding in the presence of anti- $T_4$  antiserum was  $43.4 \pm 1.0\%$  on using PEG, and  $44.3 \pm 1.4\%$  on using *S. aureus*. <sup>c</sup> Difference with respect to the values found for blanks (RIA buffer) are statistically significant ( $p < 0.05$  or better) by the paired  $t$ -test.



**Fig. 3. Comparison of found and calculated  $T_4$**

Bar A, the apparent amount of  $T_4$  in 50  $\mu$ L of whole milk, as determined by RIA. Bar B, amounts of  $T_4$  that should have been found in skimmed milk and in milk fat, as calculated from the data found in A, and the individual partition coefficients of the added  $T_4$  ( $69.9 \pm 4.9\%$  of  $T_4$  in skimmed milk and  $30.1 \pm 4.9\%$  in fat). Bar C, amounts actually found by RIA on using 50  $\mu$ L of skimmed milk or the fat in 50  $\mu$ L of whole milk, corresponding to the four samples used for Fig. 1



**Fig. 4. Upper panel:** the pattern of elution of  $T_4^*$  added to human milk, after passing it through Bio-Rad columns and the successive washes described here

Practically all of the  $T_4^*$  is eluted with acetic acid (Ach), 500 mL/L. Fractions are of 0.5-mL volume. Eluate 1 is formed by pooling the two fractions with the highest  $T_4^*$  content so that 40-80  $\mu$ L of milk equivalents will require minimum volumes of Eluate 1. The shaded area indicates fractions with high turbidity, or opalescence. Most of the "milky" components are eluted in the first washes, but the first fractions eluting with Ach are not completely clear. When the column is further washed with 700 mL/L Ach, the first eluates are again opalescent. Care is taken to choose for eluate 1 those fractions with a high  $T_4^*$  content that are most transparent. No visible residue remains in the RIA tubes after evaporation of 40-80  $\mu$ L of eluate 1

**Lower panel:** the pattern of distribution of  $T_4^*$  contained in eluate 1 after ascending paper chromatography (13)

The  $T_4^*$  in the strips indicated by the shaded area is eluted and pooled to obtain eluate 2

We observed that the apparent  $T_4$  concentration also depended on the pretreatment given the sample (Table 1): the lower apparent  $T_4$  values found for 1:2 EtOH as compared with 1:10 EtOH extracts could not be accounted for by the  $T_4$  extraction coefficient, which exceeded 91% in both cases. Thus the difference persists when the  $T_4$  concentration in whole milk is calculated; the fat also appeared to contain significantly more  $T_4$  than expected from the  $T_4$  partition coefficient (column B of Table 1). We noticed that a layer of fat was visible in 1:2 EtOH extracts of whole milk, but not in the 1:10 EtOH extracts; evidently more fat was being dissolved in 1:10 than in 1:2 EtOH extracts. Milk fat separated from whole milk by centrifugation was also completely dissolved in EtOH.

The sum of the apparent amounts of  $T_4$  found in skimmed milk and milk fat exceeds that found in whole milk (Figure 3). When the  $T_4$  partition coefficients were applied to the  $T_4$  data found for whole milk, the calculated amount of  $T_4$  and that actually measured by RIA coincided for the skimmed milk fraction. In contrast, the amount measured by RIA for the milk fat significantly exceeded the calculated one.

We considered the possibility that these problems were caused by the presence of moieties binding the labeled antigen, especially when the lipid content of the sample transferred to the RIA is high, because the use of skimmed milk did not solve all the problems encountered. To assess such possibilities we evaluated the nonspecific binding of  $T_4$ . Table 2 shows results obtained with both PEG and *S. aureus*. Nonspecific binding of  $T_4$  was often greater in samples derived from milk than in RIA buffer alone, and often appeared related to the probable fat content of the sample (column B of Table 2). Nonspecific binding by *S. aureus* could be as great as total binding in samples likely to contain a high proportion of milk fats.

The lipophilic nature of  $T_4$  made it unlikely that solvent-extraction procedures would adequately separate it from lipidic milk components. Paper chromatography was initially attempted with whole milk, skimmed milk, and EtOH extracts, but good separations were not consistently obtained. Bio-Rad resin columns (12) were used and preliminary ex-

periments done to define the appropriate volumes of milk relative to the amount of resin and the procedure that would elute most of the  $T_4$  in a clear, non-opalescent fraction. Procedure B was developed and initially validated with procedure C, which involves paper chromatography of the resin eluate.

Figure 4 shows elution patterns of  $T_4$  after its adsorption on a Bio-Rad resin column and after paper chromatography of the resin eluate. Good parallelism was found between the  $T_4$  standard curves and serial dilutions of both eluates 1 and 2 (Figure 5). The eluates from the "blank" columns did not interfere in the  $T_4$  RIA. Figure 6 confirms, for both eluates 1 and 2, that the  $T_4$  concentration is no longer dependent on the volume of sample in the RIA tube. The nonspecific binding of  $T_4$  as evaluated with eluate 2 was the same as that of the standard curve blanks (Table 3), but specific binding with eluate 1 was somewhat higher. Despite this, when we used the individual blanks to calculate the  $T_4$  concentration, the results obtained with eluates 1 and 2 were the same (Figure 6).

Individual blanks increase with the amount of eluate 1 used in the RIA. If large volumes of eluate 1 are needed because of a low  $T_4$  concentration in the milk sample, procedure C should be followed. The same is advisable if individual blanks for eluate 1 are unusually high. Procedure B should not be altered without previous validation. Thus, an increase in the volume of milk relative to that of the resin bed would decrease the proportion of  $T_4$  retained on the column and later eluted with acetic acid/water (1/1 by vol). A decrease in the amount of milk used relative to that of resin bed would result in the use of larger aliquots of eluate 1, a procedure that should be avoided for the reason indicated above.

Figure 7 shows our preliminary frequency distribution of the  $T_4$  concentrations in human milk. Most of the samples were obtained two or three weeks postpartum. As may be seen, in none of the samples tested so far did the  $T_4$  concentration exceed 2  $\mu\text{g/L}$ .

## Discussion

Our results show that human milk contains substances that interfere with  $T_4$  RIA in proportion to the volume of milk

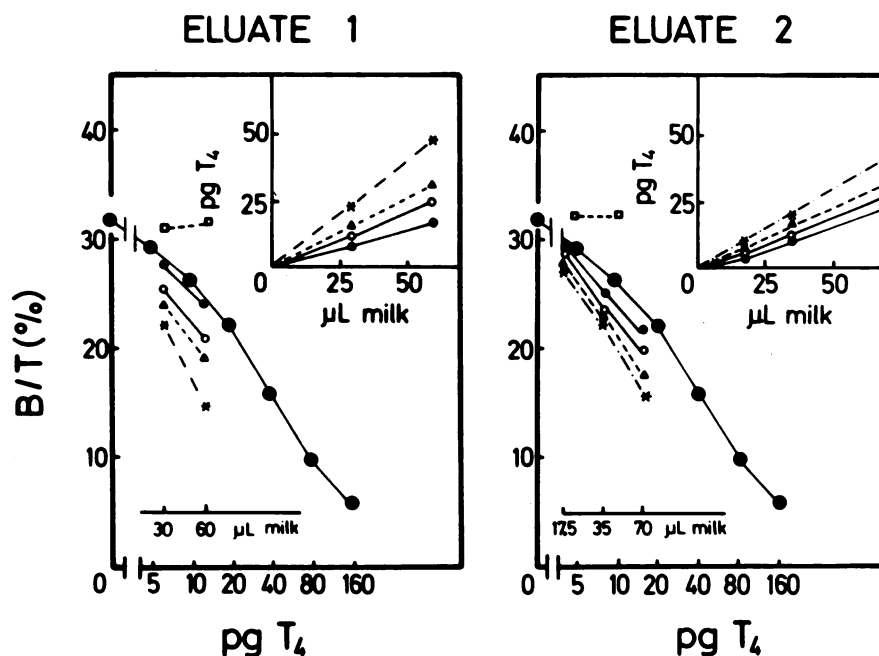


Fig. 5. Serial dilution curves obtained with eluates 1 and 2

For the sake of simplicity, the milk volumes shown are the approximate means corresponding to 40 and 80  $\mu\text{L}$  of eluate 1, or 20, 100, and 200  $\mu\text{L}$  of eluate 2, as assessed from individual  $T_4$  recoveries. The actual mean values are given in Table 3. Different symbols are used to identify milk specimens (different from those used for previous Figures and Tables). ( $\square$  - - -  $\square$ ), results obtained for eluates 1 and 2 from "blank" columns



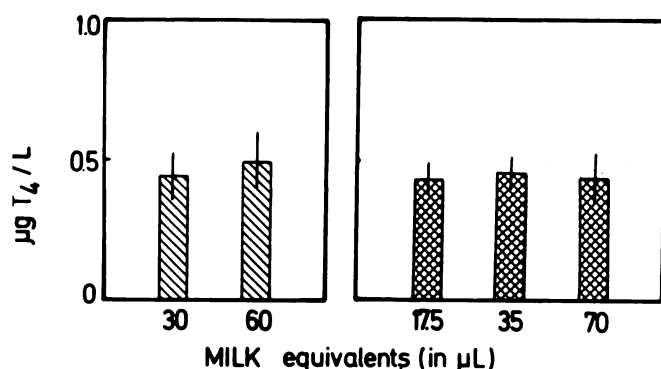


Fig. 6.  $T_4$  concentrations found on different volumes of milk equivalents for the RIA

Results no longer depend on sample volume, and are the same whether the resin eluate is further purified by paper chromatography (right, eluate 2) or not (left, eluate 1)

required for the RIA. Previous studies reporting  $T_4$  concentrations  $>5 \mu\text{g/L}$  (1–4) were done with less-sensitive competitive binding or radioimmunoassays that required larger sample volumes (100  $\mu\text{L}$  or more). Thus it is likely that those results were artifactual, a possibility apparently not excluded by the authors, because they report no validation tests, such as serial-dilution curves and the like. The use of skimmed milk or EtOH extracts as samples would not necessarily obviate these artifacts, as shown here.

Tenore et al. (16) reported that there might be analytical problems with the determination of  $T_4$  by RIA:  $T_4^*$ -displacement activity in the  $T_4$  RIA did not coincide, after chromatography on Sephadex LH 20, with the fractions eluting the radiolabeled  $T_4$  added to the milk sample as tracer. The RIA activity was eluted in the void volume, where no labeled  $T_4$  was found, whereas no RIA activity was found in the peak containing tracer  $T_4$ . In view of our results perhaps the amounts of  $T_4$  eluted in the fraction containing the tracer  $T_4$  were too small to be detected by RIA, and the “radioimmunoassayable activity” they found in the void volume actually reflected the presence of moieties interfering in the  $T_4$  RIA.

The interpretation suggested by these authors was, however, that endogenous  $T_4$  did not equilibrate with the added labeled  $T_4$ . If this were so, values obtained with our procedures B and C might be underestimated. But we believe that the data in Figure 3 show this to be unlikely, because the amount of  $T_4$  in the skimmed-milk fraction coincided with the one calculated from the whole-milk  $T_4$  content and the  $T_4^*$  partition coefficient. In contrast, the amount of  $T_4$  in the fat layer

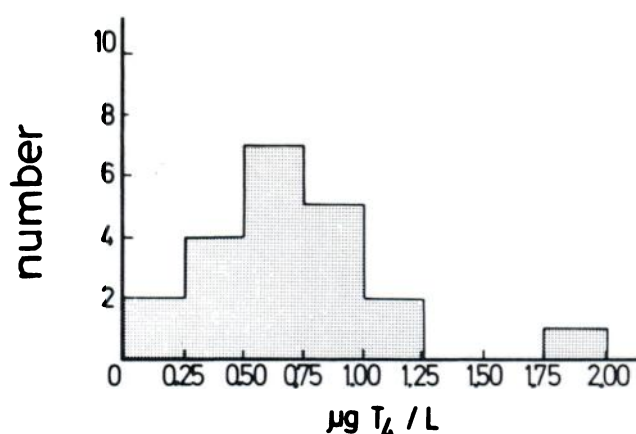


Fig. 7. Distribution curve of  $T_4$  concentration found in 21 samples of human milk, most of which were obtained two to three weeks postpartum

The mean value ( $\pm$ SEM) was  $0.71 \pm 0.09$  (range 0.24–2.00)  $\mu\text{g/L}$

did not correlate, probably because of the presence of interfering moieties. The sum of the  $T_4$  found in the skimmed milk plus that in the fat layer should have been the same as the  $T_4$  found in whole milk if the only problem were a lack of equilibration of added  $T_4^*$  with the endogenous  $T_4$ .

Apart from interferences apparently related to its high lipid content, human milk might contain thyroxine-binding proteins; corticosteroid-binding globulins have been identified in human and rat milk, and are apparently identical to the binding proteins in serum (17, 18). This might also affect the RIA results, because the amounts of “blockers” added for the determination of  $T_4$  in serum might not be adequate.

Procedures B and C are currently being validated for the determination of  $T_4$  in sera and tissues with a high lipid content.

Our results are in essential agreement with the reports by Varma et al. (5), Sato and Suzuki (6), and Tsuyusaki et al. (7). In view of the results by Sato and Suzuki (6) for trypsin digests of human milk, it is unlikely that the total  $T_4$  content exceeds 5  $\mu\text{g}$  of  $T_4$  per liter. Thus, unless human milk contains high concentrations of  $T_3$ , which is unlikely (6), the thyroid hormone content of human milk would not afford adequate protection to the developing brain of athyreotic babies.

The reported improved mental development of breast-fed as compared with formula-fed hypothyroid babies (4) might be related to some other nutritional difference, such as the iodine content (19). The human baby depends totally on the iodine in milk for thyroid function during the suckling period; ectopic thyroid tissue might function longer if the iodine supply is adequate.

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**Table 3. Binding of  $T_4^*$  in the Absence of Anti- $T_4$  Antiserum in RIA Tubes Containing Different Volumes of Eluates 1 and 2 from Seven Different Milk Specimens <sup>a</sup>**

Sample	Eluate evaporated, $\mu\text{L}$	$\mu\text{L}$ of milk equivalent <sup>b</sup>	% of $T_4^*$ (nonspecific binding)
RIA buffer <sup>c</sup>	—	—	7.0 (0.2)
Eluate 1	40	28.4 (4.2)	8.5 (0.5) <sup>d</sup>
Eluate 1	80	56.8 (4.2)	10.0 (0.4) <sup>d</sup>
Eluate 2	50	17.8 (5.9)	7.1 (0.1)
Eluate 2	100	35.7 (5.9)	7.4 (0.5)
Eluate 2	200	71.4 (5.9)	7.8 (1.3)

<sup>a</sup> Data are means (and SEM), with separation by PEG. <sup>b</sup> The mean (and SEM) of the equivalent amounts of milk, based on the individual  $T_4^*$  recovery data. <sup>c</sup> The total binding in the presence of anti- $T_4$  antibody was  $39.1 \pm 0.4\%$ . <sup>d</sup> Difference with respect to the RIA buffer (standard curve blanks) is statistically significant.

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